Appendix E

COMPARISON BETWEEN WILD TYPE AND MUTATED RECEPTORS: TDAG & GPR35

A. TDAG8

293 cells were plated-out on 150mm plates at a density of 1.3 x 10^7 cells per plate, and were transfected using 12μg of the respective DNA and 60μl of Lipofectamine Reagent (BRL) per plate. The transfected cells were grown in media containing serum for an assay performed 24 hours post-transfection.

A commercially-available, 96-well Adenylyl Cyclase Activation Flashplate™ was used (NEN: #SMP004A). First, 50μl of the standards for the assay were added to the plate, in duplicate, ranging from concentrations of 50pmol to zero pmol cAMP per well. The standard cAMP (NEN: #SMP004A) was reconstituted in water, and serial dilutions were made using 1xPBS (Irvine Scientific: #9240). Next, 50μl of the stimulation buffer (NEN: #SMP004A) was added to all wells. Next, the 293 cells transfected with the respective cDNA (CMV[1] or TDAG8) were harvested 24 hours later (assay detection in serum media). The media was aspirated and the cells washed once with 1xPBS. Then, 5ml of 1xPBS was added to the cells along with 3ml of cell dissociation buffer (Sigma: #C-1544). The detached cells were transferred to a centrifuge tube and centrifuged at room temperature for five minutes. The supernatant was removed and the cell pellet was resuspended in an appropriate amount of 1xPBS to obtain a final concentration of 2x10^6 cells per milliliter. The detection buffer containing the tracer cAMP was prepared. In 11ml of detection buffer (NEN: #SMP004A), 50μl (equal to 1μCi) of [^{125}I]cAMP (NEN: #SMP004A) was added. Following incubation, 50μl of this detection buffer containing

[1] This is the vector used for expression, such that the control was the vector, CMV, excluding the receptor TDAG8 (or GPR35 in the example below).